STIC-ILL

N° 355,118

From: Kerr, Janet
Sent: Thursday, July 12, 2001 1:20 PM
To: STIC-ILL
Subject: references for 09/457,931

Please order the following references:

Schleger et al.
Altex
18(1):5-8
2001

Prieto, P.
Science Of The Total Environment
247(2-3):349-354
2000

3728421

Genschow et al.
In Vitro & Molecular Toxicology
13(1):51-66
2000

Scholz et al.
Cells Tissues Organs
165(3-4):203-211
1999

Spielmann et al.
In Vitro Toxicology: Journal of Molecular and Cellular Toxicology
10(1):119-127
1997

Mummery et al.
Reproductive Toxicology
7(Suppl. 1):145-154
1993

Craig et al.
Biomarkers
1(2):123-135
1996

Webster, W.S.
Congenital Anom.
28(4):295-302
1989

Thanks,
Janet M. Kerr
A.U. 1633
305-4055
CM1-12A03

Reproductive Toxicology, Vol. 7, pp. 145–154, 1993
Printed in the U.S.A.

0890-6238/93 $6.00 + .00

Developmental Toxicity

REGULATION OF GROWTH AND DIFFERENTIATION IN EARLY DEVELOPMENT: OF MICE AND MODELS

CHRISTINE L. MUMMERY, HARMEN G. SLAGER, WOUTER VAN INZEN, ERIC FREUND, and ADRIANA J.M. VAN DEN EIJNDEN-VAN RAAIJ
Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Utrecht, The Netherlands

Abstract — In this article we describe some of the fundamental processes occurring during early murine development, introduce cellular models used to investigate these processes and review some well-known factors that may be involved in their control. These include transforming growth factor β, retinoic acid and leukaemia inhibitory factor. Refinements to the culture conditions of embryonic stem and embryonal carcinoma cells have enabled us to test the effects of these factors on growth and differentiation and in particular to establish that their interaction may determine the ultimate developmental state of the cell population. Preliminary studies using neutralizing antibodies in embryos are described that suggest that deregulation of normal expression can lead to a failure to implant. Insights into the events underlying normal embryonic development and implantation, yielded by the type of study described here, may contribute to an understanding of the mechanisms causing early embryonic loss and the role of toxicants in this process.

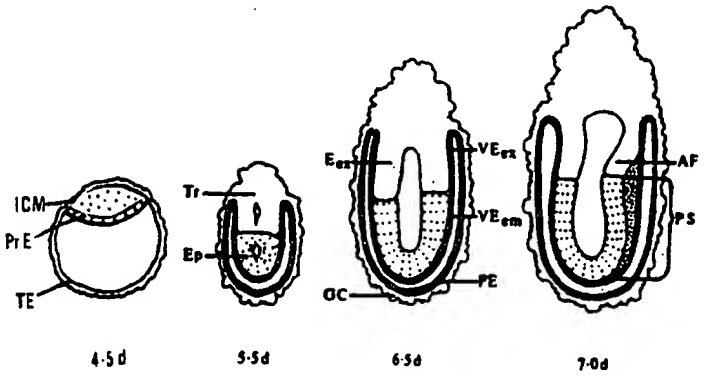
Key Words: mouse embryo; embryonal carcinoma cells; embryonic stem cells; differentiation; TGFβ; retinoic acid; leukaemia inhibitory factor.

INTRODUCTION

From one-to-five thousand cells

Following fertilization of a mouse oocyte a series of discrete interrelated events occurs that eventually leads to the formation of the three primary germ layers, that are the origin of both the embryo proper, and most of the extraembryonic tissues. Initial events, such as cell cleavage where the cells divide and become progressively smaller, occur in the oviduct as the embryo encased in a protein "shell" known as the zona pellucida (z.p.) passes towards the uterus. Between the 4- and 16-cell stage, a process known as compaction begins, where the individual blastomeres flatten, increase their contact with one another and develop distinct apical and basal membranes (polarization). The first restriction in the developmental potency of cells then occurs, the outer cells forming the trophectoderm (TE) lineage and the inner cells, the inner cell mass (ICM). The trophectoderm has features of a true epithelium, forming tight junctions, an asymmetrical distribution of the Na^+/K^+ pump and a complete seal against the outside environment. A fluid filled cavity, the blastocoele begins to form and expand until the embryo consists of a hollow vesicle of trophectoderm cells and a small group of eccentrically located ICM cells. The mural trophectoderm cells, surrounding the blastocoele cavity, cease division and become large and polyploid while those remaining in contact with the ICM continue to divide rapidly. As the embryo hatches from the z.p. and prepares for implantation, a second round of differentiation occurs: the formation of primitive endoderm and ectoderm. This is characterized by the appearance of an epithelial layer—the primitive endoderm—on the surface of the ICM facing the blastocoelic cavity (Figure 1). A thin basal lamina forms on the apical side of the trophectoderm over which some cells of the primitive endoderm migrate, differentiating to form the parietal endoderm of the yolk sac as they proceed. These cells are characterized by enormous production and secretion of extracellular matrix (ECM) proteins that form the Reichert's membrane surrounding the embryo. Cells remaining in contact with the ICM differentiate to form the visceral endoderm cells of the yolk sac, separated from the ICM by a basal lamina and distinctive on the basis of alpha-foetoprotein expression, microvilli on the cell

Address correspondence to Christine L. Mummery, Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.



ICM : Inner cell mass
Pr E : Primitive Endoderm
TE : Trophectoderm
TR : Trophoblast
Ep : Epiblast
Eex : Extraembryonic ectoderm
GC : Giant cells
VEex : Extraembryonic visceral endoderm
VEem : Embryonic visceral endoderm
PE : Parietal endoderm
AF : Amniotic fold
PS : Primitive streak

Fig. 1. Earliest differentiation events and the formation of three germ layers in the murine embryo. After Hogan et al. (66).

surface, and high levels of endocytotic activity; these characteristics are typical of cells with a nutritive function. Finally at about 6.5 days of gestation, cells delaminate by an unknown mechanism from the epithelial layer of the primitive ectoderm and accumulate as a layer of individual mesoderm cells between the primitive ectoderm and the visceral endoderm. The primitive ectoderm lineage gives rise to the ectodermal, mesodermal and endodermal tissues of the fetus, to the germ cells and to the mesodermal components of the extra embryonic membranes and placenta (1).

In the first third of the gestation period up to early implantation therefore, the embryo develops from a single cell to a complex structure with three germ layers set aside. Despite this fairly thorough description of the morphologic changes that occur however, little is known about the orchestrated interactions between cellular groups. Exceptionally, it has been shown that the cell adhesion molecule uvomorulin is at least in part responsible for compaction since anti-uvomorulin antibodies inhibit this process (2) while hatching is brought about by a trypsin-like enzyme synthesized by cells in the mural trophoblast (3).

Peptide growth factors, retinoids, and ECM proteins with their respective receptors are also prime candidates as mediators of cell-to-cell interactions in the early embryo on the basis of their differential expression in time and space and their known effects on various cell model systems in vitro [reviewed (4)]. Using an approach based on the study of embryonal carcinoma (EC) and embryonic stem (ES) cells, which resemble the pluripotent cells of the ICM and can form many of its direct descendants in vitro, we are rapidly gaining new insights into fundamental processes such as the initiation and direction of differentiation, the origin and function of the first basal laminae, and the regulation of cell proliferation. Interference with any of these processes may lead to the failure of an embryo to develop and implant; understanding the regulation of the processes themselves is essential to understanding how potentially embryotoxic substances might work and whether their effects could be predicted or even prevented. In what follows, we will consider the roles and interactions of three particular factors, namely transforming growth factor β (TGFβ), retinoic acid (RA) and leukaemia inhibiting factor (LIF).

Transforming Growth Factor β in Early Development

TGFβ was originally purified from human platelets as a 25 kDa homodimeric protein based on its ability to induce anchorage-independent growth of normal fibroblast indicator cells (5). It was later identified in many non-neoplastic tissues, transformed cells and in media conditioned by many cell lines in a latent biologic form (6,7) and found to act as a mitogen, growth inhibitor, or regulator of differentiation upon activation depending on the nature of the target cell (8). It is now apparent that at least five genetically distinct forms exist (TGF$\beta_{1\text{-}5}$), three of which have been cloned in mammals. They have extensive amino acid homology and to date few functional differences have been described between

them. Exceptionally, TGFβ_2 has been shown to induce dorsal mesodermal tissues in explants of Xenopus laevis embryos while TGFβ_1 is inactive (9). TGFβ_3 is ten times more potent than TGFβ_2 in this assay (10). All three isoforms are differentially regulated in differentiating EC and ES cells (11,12). EC and ES cells only express TGFβ_1 but after differentiation, TGFβ_2 is expressed as well as TGFβ_3 in some mesenchymal derivatives (11,12). TGFβ_1 can inhibit the expression of protein markers of parietal endoderm cells, including secretion of the ECM protein laminin, and may be an inhibitor of the process primitive-to-parietal endoderm (13). In the preimplantation embryo TGFβ_1 and β_2 mRNA and/or protein have been described from the 4-cell stage up to blastocyst formation (14–16). TGFβ_2 protein is restricted to the trophectoderm at the blastocyst stage. In later development TGFβ_1 mRNA is expressed in endothelial tissues (17) and TGFβ_1 and β_2 mRNA in uterine decidua at 6.5 days of gestation (18), which would be compatible with a role in mesoderm formation in the mouse as well as amphibian. This still requires formal proof. Neither TGFβ_1 mRNA or β_2 was detected by in situ hybridization in the embryo itself at 6.5 days in this latter study although we have clearly detected protein then in visceral endoderm (Figure 2 in reference 16). It remains to be established whether this was taken up by receptor-mediated processes in these cells or whether the level of mRNA was below the detection limit of the technique. Differences between protein and mRNA localization for TGFβ have previously been observed in the spleen (19) and embryonic heart (20).

In terms of effects of TGFβ on developing murine embryos, it has recently been shown that TGFβ_1 can enhance the rate of blastocyst formation of embryos cultured singly in drops of medium to a level equivalent to that of embryos grown in groups in close proximity. Specific membrane receptors for TGFβ are then presumably present in preimplantation embryos although this remains to be established. Two of the three receptors for TGFβ (the type II and III receptor) have recently been cloned (21,22). This provides an immediate strategy for characterizing receptor expression in the embryo. On the basis of binding studies in EC and ES cells (4), receptor expression would not be expected in the ICM but would be in its endodermal and mesodermal derivatives.

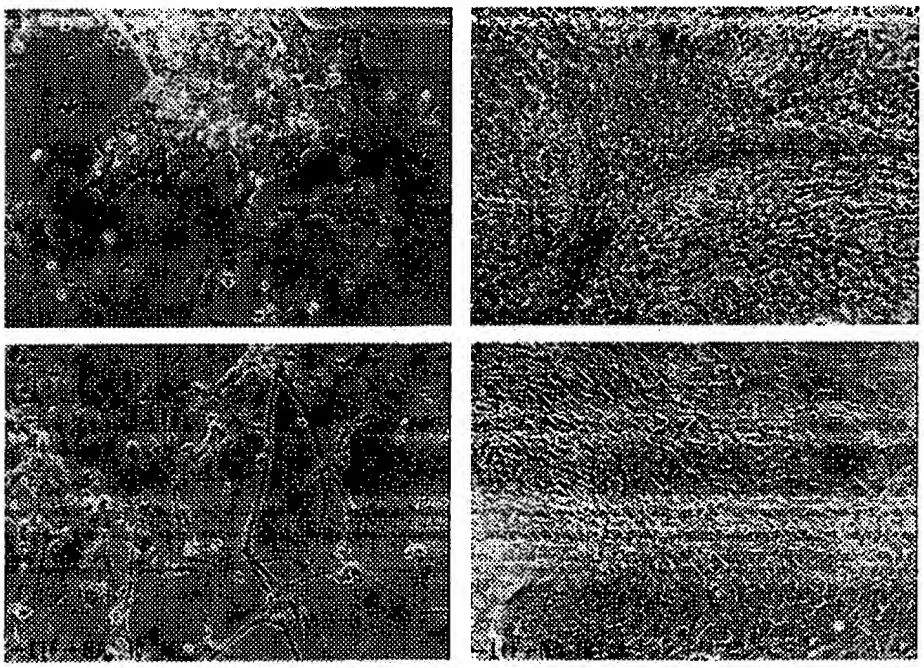


Fig. 2. Induction of differentiation in ES cell aggregates by retinoic acid. Cells were induced to form a single aggregate in hanging drops in the presence of the concentrations of retinoic acid indicated, as described in Methods. After 10 days, the aggregates were plated on tissue culture plastic and the phenotypes of the differentiated cells present scored on day 16.

Retinoic Acid in Development

Retinoids are widely known for their teratogenic effects on almost all species tested, including humans. Head and face malformations are induced in mice, head defects in rats, face and digital anomalies in rabbits, jaw and tongue defects in guinea pigs, eye abnormalities in swine, and multiple defects in dogs and in hamsters (reviewed in 23,24). The mechanism by which these congenital malformations are induced is unknown but disruption of cell proliferation in epithelial–mesodermal tissues appears to be the origin of at least the facial clefts in rats (25). It has recently become clear, however, that in addition to these pharmacologic effects, retinoids may have a physiological function in development. Indeed, retinoids appear to possess the properties of classical "morphogens," diffusible substances that determine cell fate in a concentration dependent way. In the chick limb bud, transplantation of a piece of posterior wing bud mesenchyme to an anterior position in a host wing results in a mirror-image symmetric duplication of the host's wing pattern (26). The effects of this "zone of polarizing activity" (ZPA) can be mimicked by retinoic acid; implantation of an anion exchange bead soaked in RA at the anterior limb bud margin induces duplications similar to those induced by ZPA in a dose dependent way (27,28). It later turned out that both chick and mouse limb buds actually contain endogenous retinoids (29,30) and that RA is enriched 2.5 fold in the ZPA-containing region (29). Such a shallow gradient of total RA suggests that cells distinguish sharply between relatively small differences in RA although there may be local mechanisms, such as the presence of cellular retinoic acid binding proteins (CRABP), which amplify the gradient. Maden (31) and Dollé (32) found that CRABP is in fact enriched in the anterior limb bud region, that is opposite to the ZPA; CRABP may thus regulate the relative amounts of bound versus free RA. In turn, free RA may be involved in regulating the expression pattern of various genes, including those for the nuclear receptors for RA (RARs) themselves. These are known to be spatially distributed in the early developing vertebrate limb [reviewed by Eichele (33)].

Four different RAR cDNAs have been identified in mammals, three of which (RARα, β, and γ) are closely related and are known to exist in several isoforms [reviewed (34,35)]. It has been shown that RARα and γ but not RARβ are expressed in undifferentiated EC and ES cells but that RARβ is strikingly and rapidly induced within a few hours of RA addition (36–38). This ultimately results in cell differentiation, the cell types formed depending on the local cell density at the time of RA addition. F9 EC and P19 EC cells for example will form endodermlike cells when 10^{-6} M RA [± dbcAMP] is added to cultures in monolayer (12,39,40) but visceral endoderm-like cells and neural derivatives respectively when the same concentrations are added to cells aggregated in suspension.

The RARs have a common general structure, with homologous regions, the DNA-binding (C-) domain and the hormone-(ligand) binding (E-) domain present in all family members [reviewed (41)]. Following binding of RA, the C-domain becomes capable of binding to DNA on specific target genes, thereby regulating their expression. RARβ gene expression is regulated by three alternative promotors leading to at least three different isoforms of RARβ (42). It is the RARβ_2 promotor that is predominantly active in RA-treated EC and ES cells.

It has recently been demonstrated that RA is also a putative morphogen in the amphibian Xenopus laevis (43). Treating early Xenopus embryos with RA causes microcephaly, the result of transformation of anterior neural tissue in the developing central nervous system to a posterior neural specification. The microcephalic embryos lack most or all of the forebrain and midbrain as well as the sense organs (eyes and nasal pits) that are induced by or formed from the forebrain. The embryonic stages sensitive to RA were also shown to contain endogenous RA at concentrations similar to the highest reported in the chick limb bud. It was subsequently shown that early Xenopus embryos apparently contain an RA-sensitive anterior "domain" that transcends germ layers and includes both ectoderm and mesoderm (44). RA appears to have profound effects on anteroposterior axis specification and may thus play a major role in the formation of the primary body axis.

LIF in Early Development

Leukaemia Inhibitory Factor (LIF) is a secreted glycoprotein that is a member of the cytokine group of proteins that regulate the proliferation and differentiation of many eukaryotic cells. As other members of the group, LIF was initially thought to have only a limited set of target cells but it is becoming evident that a wide variety of tissues both in the embryo and in the adult organism respond to or secrete LIF. Originally identified in medium conditioned by Krebs II ascites cells, it was characterized, purified, and cloned on the basis of its ability to in-

duce macrophage differentiation in M_1 murine myeloid leukaemia cells and suppress their proliferation in vitro (45–48). One of the most unexpected properties of LIF was the ability to induce opposite biological effects in different target cells; the differentiation of particular EC cell lines and ES cells, historically established and maintained on embryonic fibroblast feeder cells (49,50) was entirely inhibited in monolayer culture by LIF (51). It was already known that conditioned medium from some feeder cells, particularly Buffalo rat liver (BRL) cells, could replace the requirements for feeders in monolayer culture although differentiation to visceral endoderm but not to mesoderm still occurred on embryonic bodies formed in conditioned medium (52). This permissiveness to endoderm differentiation later appeared to be more general since cells expressing parietal endoderm markers formed when RA was added to ES cells in monolayer culture even in the presence of BRL conditioned medium (12,40). Purification and partial amino acid sequencing demonstrated that this differentiation inhibiting activity (DIA) secreted by BRL cells was in fact identical to LIF (53) and to human interleukin for DA-1a cells (54). It was subsequently demonstrated that pluripotent cell lines could not only be propagated in the absence of feeder cells in medium containing recombinant LIF but could also be established directly from embryos under these conditions (55). The lines thus derived were karyotypically normal and retained their ability to differentiate in vitro in the absence of LIF and in vivo when injected into host blastocysts. Even after 2 months of continuous passage, or approximately 100 cell generations, in medium containing recombinant LIF, ES cells were shown to retain their full developmental potential and to be able to contribute efficiently to the germ line of chimaeras (56).

Although it had been formally demonstrated that LIF and DIA in BRL-cell conditioned medium (BRL-CM) were identical, the secretion of LIF into the medium by other feeder cells appeared to be insufficient to explain their ability to inhibit ES cell differentiation. The DIA/LIF activity of these feeder cells has recently been shown to be substantially localized in the extracellular matrix; matrix extracts from these cells inhibit ES cell differentiation entirely (57). By alternative first exon usage the amino terminus of the primary translation product is changed to direct incorporation of mature, biologically active DIA/LIF into the extracellular matrix or into the extracellular supernatant.

There are important distinctions between the roles of diffusible and immobilized signalling molecules, particularly in development. Diffusible factors can mediate communication between nonadjacent cells, give rise to concentration gradients, and effect cellular responses on the basis of receptor expression rather than spatial location. Immobilized factors only affect adjacent cells, provide a means of limiting signals to particular sites and possibly of storing signals for future use. To what extent these variants of LIF exist in vivo and play a role in the biological effects is at present unknown. In the developing mouse embryo, LIF transcripts are detectable as early as the blastocyst stage at 3.5 days p.c. (58) and at least up to 12.5 days p.c. but are restricted to extraembryonic tissues. The inner cell mass and ES cells do not express LIF. It could be speculated that in the developing embryo the diffusible form of LIF, for example from the trophectoderm of the blastocyst or uterine endometrial glands (59), may be involved in maintaining pluripotentiality in a three dimensional cell population such as the ICM, while the matrix associated form may be involved at a later stage maintaining pluripotentiality in a stem cell monolayer such as the primitive ectoderm (57). Which cell types in the embryo express specific receptors for LIF is at present unknown although ES cells have been shown to express similar receptor levels (53) as a number of hemopoietic cell types (~300 per cell) (60).

The multifunctionality of LIF and the diversity in its sources, biological effects, and target cells, clearly demonstrate its importance as a regulator of growth and differentiation in normal adult tissues and the developing embryo; its possible role in tumorigenesis is, however, less clear. In the case of teratocarcinomas, where differentiation is accompanied by the loss of malignant phenotype and the development of a benign teratoma, LIF produced by surrounding (differentiated) "feeder" cells may be involved in maintaining the malignant stem cell population in the undifferentiated state. Conversely, its ability to act as a negative regulator of growth and induce differentiation of leukaemia cells in this case results in the loss of the malignant phenotype. Understanding the regulation of the expression of both the ligand and receptor for LIF may lead to possible new routes for therapy.

It is clear that the particular factors described above are highly implicated as regulators of early murine development. We will now describe some recent results obtained using ES cells under newly defined conditions that bring us closer to understanding their exact function.

MATERIALS AND METHODS

Cell culture

ES cells were cultured as previously (11,12) in 80% BRL-CM with 20% MEM, plus 20% FCS; cells were passaged 1:5 twice weekly using trypsin/EDTA and not used beyond 15 passages from transfer from feeder layers to BRL-CM.

DCC-FCS

The protocol for the preparation of charcoal-stripped fetal calf serum has been described previously (61).

Hanging drop assay

This has been described previously for P19 EC cells (61,62). In brief, cells were trypsinized as above, resuspended in MEM with 20% DCC-FCS and β-mercaptoethanol, and diluted to a density of 40,000 cell/mL. 20 μL drops containing 800 cells were placed on the lid of a bacterial grade Petri dish, and this inverted over the dish itself containing phosphate buffered saline. The cells thus form a single aggregate. RA/LIF were added as required and medium refreshed every three days. Individual aggregates were plated on gelatinized tissue culture wells on day 10 and scored for differentiation on day 16.

Immunofluorescence

Cells were fixed at the time point required using 2% paraformaldehyde/0.1% glutaraldehyde, as previously (16). They were stained with anti-MF20, that recognizes muscle specific myosin and the appropriate FITC-labelled secondary antibody, as previously. (40,61).

Antibody injection

Neutralizing anti-TGFβ_2 antibodies were injected in blastocyst stage embryos (3.5 days p.c.) that were permitted to recover for 16 h before transfer to pseudopregnant females. Implanted embryos were scored on the equivalent of day 8.

RESULTS

Effects of RA

We have shown previously that DCC treatment of FCS removes lipophilic substances, which would include retinoids, from FCS and that using this serum to supplement culture media eliminates "spontaneous" differentiation of P19 EC cells (61,62). Similar treatment of ES cell aggregates renders them exquisitely sensitive to RA, as shown in Figure 2. In control cultures without RA and at 10^{-9} M RA, extensive areas of endoderm and mesoderm-like cells, including beating muscle, are observed at day 16, while at 10^{-8} M RA, extensive fields of neurites (50 or more) emerge from the plated aggregates. At 10^{-7} M RA, neurites also form, together with endoderm cells and endothelial vessels (Figure 2). At the higher RA concentrations, the formation of beating muscle cells and mononucleate flat cells that stain with anti-MF-20 is completely repressed although the formation of (nonbeating) myoblasts is slightly increased. At 10^{-6} M RA, a concentration frequently used to induce differentiation in conventional serum containing medium, the majority of cells die; the toxic dose is significantly altered under these conditions.

Effects of LIF

If LIF is present during the aggregation phase of the experiments described above, all differentiation is blocked, except that to endoderm (Figure 3), independent of the concentration of RA present. In all cases cells with an epithelial morphology typical of (parietal) endoderm are formed.

Effects of TGFβ

Preliminary experiments show that in this assay TGFβ promotes the formation of mesoderm as evidenced by the number of regions of beating muscle cells and the extent of staining by anti-MF 20 in immunofluorescence in the culture at day 16 (data not shown). TGFβ_2 in particular consistently increases the rate at which beating muscle appears in the cultures, while all three isoforms dramatically increase the number of myoblasts and mononucleate flat cells staining with anti-MF-20 (5 to 7 fold) at day 16. Although the number of myoblasts formed under these conditions is not affected by RA (10^{-9} M), the formation of mononucleate flat cells and beating muscle is completely repressed.

Effects of neutralizing antibodies in the embryo

Interpretation of this type of experiment is complicated since it is difficult to estimate the volume of antibody microinjected, and unclear exactly what concentrations of antibody would be sufficient to neutralize TGFβ activity in the blastocoelic cavity. However, we have been able to show consistently in pilot studies (63) that in experiments where blastocysts injected with anti-TGFβ_2 antibody are transferred to one uterine horn of a pseudopregnant female, some 40% implant with 27% resorptions, while for control blastocysts injected with control IgG and transferred to the other horn, about 90% implant and only 19% are resorbed.

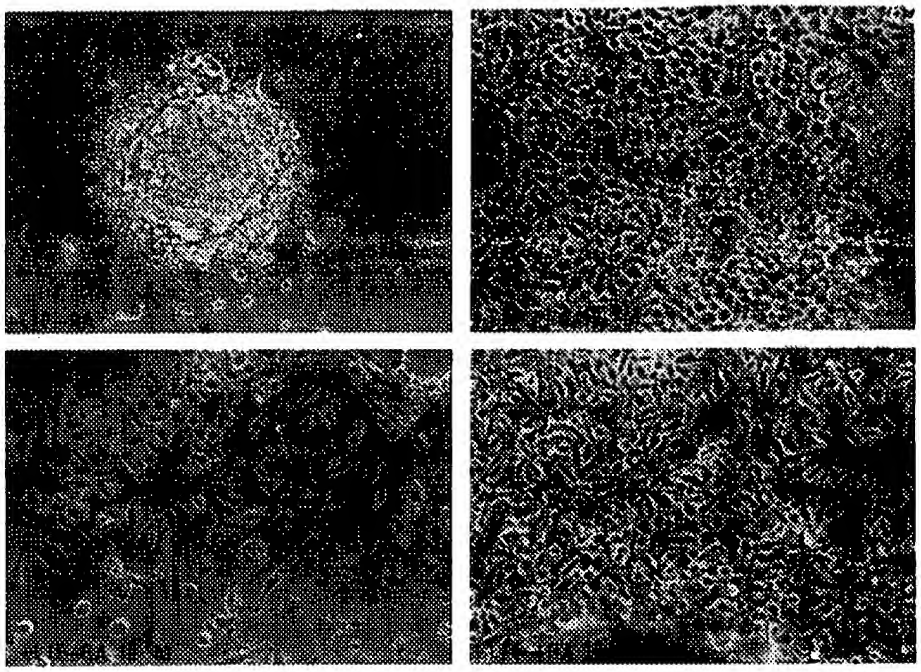


Fig. 3. Effect of LIF on the RA-induced differentiation of ES cell aggregates. Cells were induced to differentiate as in the legend to Figure 2 but were exposed to LIF during the aggregation period.

DISCUSSION

The results using ES cells under more defined culture conditions than usually applied have demonstrated that interactions exist between differentiation inducing and inhibiting factors and that it is probably the subtle balance between these factors that determine the final state of differentiation of a particular cell in the embryo (Figure 4). As in the amphibian, it is likely that TGFβ plays a role in mesoderm differentiation possibly in differentiation in the heart, yet on another target cell in the embryo (primitive endoderm) it may actually inhibit differentiation. Thus it is not only the nature of the factor but also that of the cell receiving the signal that determines a final effect. Identification of cells expressing receptors for these factors will certainly lead to a further examination of differences in signal transduction pathways. Whether it is via an effect on differentiation that embryos fail to implant after introduction of neutralizing antibodies remains to be investigated.

LIF would also appear to be important for normal early development. Although not expressed in the embryo, it is expressed in maternal decidua at an appropriate moment in development when it may regulate locally which cells form endoderm. Further, we have evidence that cells of the ICM secrete laminin that is deposited on the basal side of the trophectoderm coincidently with its own receptor, α_6 integrin (64). This in turn probably forms the substrate over which newly emerging parietal endoderm can migrate. TGFβ may well regulate α_6 integrin expression. Alternatively, or in addition, TGFβ may regulate levels of fibronectin. Visceral endoderm cells will convert to parietal endoderm when in contact with fibronectin (65); this may provide an indirect means by which TGFβ regulates differentiation.

The molecular basis for the differential biological effects of RA described are not yet clear but we assume there is some differential regulation of the nuclear receptors. The ability to induce differentiation of EC cells to endoderm has long been known (39) as is the ability of excess RA to induce teratogenic effects on embryos, but these more subtle effects on ES cells suggest that retinoids may also have a role in normal development.

Although not directly applicable to toxicology and/or teratology, the approach described here investigating fundamental processes such as cell division, differentiation, morphogenesis, and implantation of the embryo, may yield new insights into the mechanisms of abnormal development. Early embryonic loss in particular is a frequent, yet often unexplained, occurrence. Chromosomal abnormalities have often been found in cases where embryonic

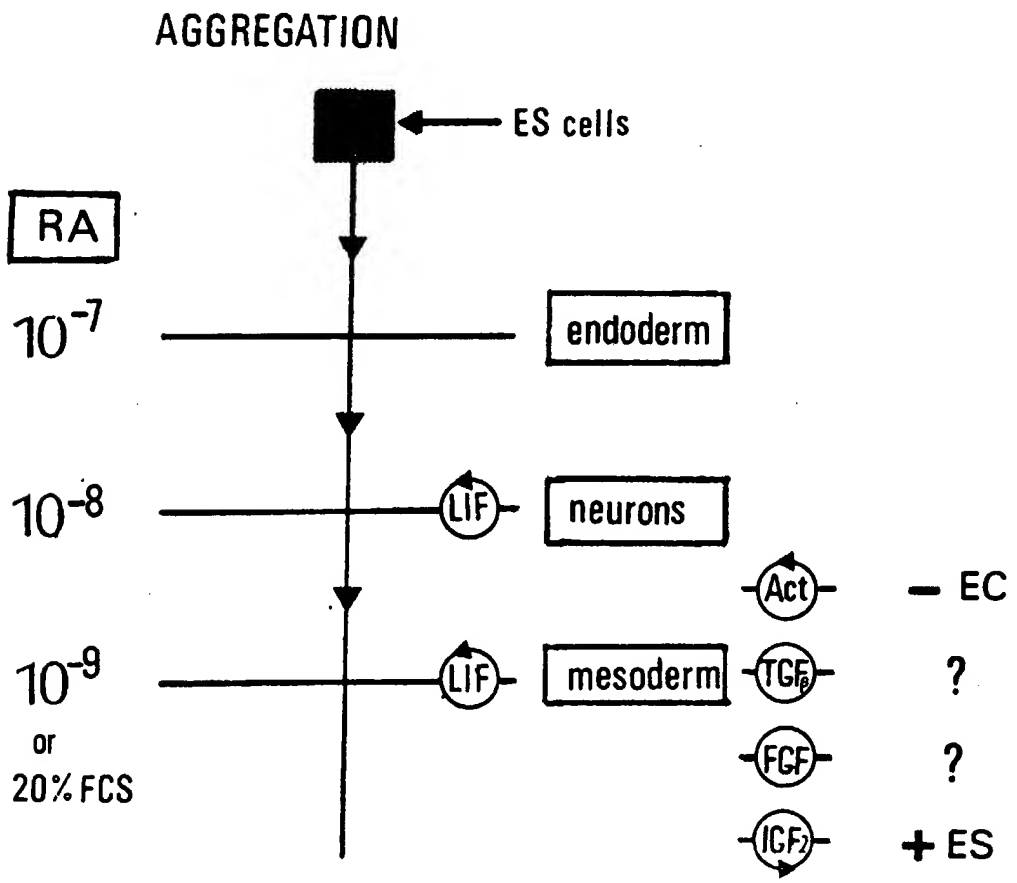


Fig. 4. The regulation of ES cell differentiation by the interaction of RA, LIF, and growth factors.

cells could still be recovered but this is unlikely to be the cause of all reproductive failure. Toxic substances could result in the death of rapidly dividing cells of the embryo or affect the delicate mechanisms involved in the initial attachment of embryos to the uterine wall and their subsequent invasion of maternal tissue.

Acknowledgments — We thank Alie Feijen for her contribution to these studies and the secretarial staff of the Hubrecht Laboratory for typing the manuscript.

REFERENCES

1. Gardner RL, Rossant J. Investigation of the fate of 4.5 d post coitum mouse ICM cells by blastocyst injection. J Embryol Exp Morphol. 1979;52:141–52.
2. Kemler R, Babinet C, Eisen H, Jacob F. Surface antigen in early differentiation. Proc Natl Acad Sci. 1977;74:4449–52.
3. Wassarman PM, Greve JM, Perona RM, Roller RJ, Salzmann GS. How mouse eggs put on and take off their extracellular coat. In: Davidson E, Firtel R, eds. Molecular biology of development. New York: Alan R. Liss; 1984.
4. Mummery CL, van den Eijnden-van Raaij AJM. Growth factors and their receptors in differentiation and early murine development. Cell Diff Dev. 1990;30:1–18.
5. Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB. Transforming growth factor-beta in human platelets: identification of a major storage site, purification and characterization. J Biol Chem. 1983;258:7155–60.
6. Goustin AS, Leof EB, Shipley GD, Moses HL. Growth factors and cancer. Cancer Res. 1986;46:1015–29.
7. Sporn MB, Roberts AB, Wakefield LM, de Crombrugghe B. Some recent advances in the chemistry and biology of transforming growth factor-beta. J Cell Biol. 1987;105: 1039–45.
8. Massague J. The TGF-β family of growth and differentiation factors. Cell. 1987;49:437–8.
9. Rosa F, Roberts AB, Danielpour D, Dart LL, Sporn MB, Dawid IB. Mesoderm induction in amphibians: the role of TGF-β_2-like factors. Science. 1988;239:783–5.
10. Roberts AB, Kondaiah P, Rosa R, et al. Mesoderm induction in Xenopus laevis distinguishes between the various TGFβ isoforms. Growth Factors. 1990;3:277–86.
11. Mummery CL, van den Eijnden-van Raaij AJM, Feijen A, et al. Expression of growth factors during the differentiation of embryonic stem cells in monolayer. Dev Biol. 1990;142: 406–13.
12. Mummery CL, Slager H, Krujjer W, et al. Expression of transforming growth factor β_2 during the differentiation of murine embryonal carcinoma and embryonic stem cells. Dev Biol. 1990;137:161–70.
13. Kelly D, Rizzino A. Inhibitory effects of transforming growth factor β on laminin production and growth exhibited by endoderm cells derived from embryonal carcinoma cells. Differentiation. 1989;41:34–41.
14. Rappolee D, Brenner CA, Schultz R, Mark D, Werb Z. Devel-

opmental expression of PDGF, TGFα and TGF-β genes in preimplantation mouse embryos. Science. 1988;241:1823–5.
15. Kelly D, Campbell J, Tiesman I, Rizzino A. Regulation and expression of transforming growth factor β during early mammalian development. Cytotechnology. 1990;4:227–42.
16. Slager HG, Lawson KA, van den Eijnden-van Raaij AJM, de Laat SW, Mummery CL. Differential localization of TGF-β_2 in mouse preimplantation and early postimplantation development. Dev Biol. 1991;145:205–18.
17. Akhurst RF, Lehnert SA, Faissner A, Duffie E. TGF beta in murine morphogenetic processes: the early embryo and cardiogenesis. Development. 1990;108:645–56.
18. Manova K, Paynton BV, Bachvarova RF. Expression of activins of TGFβ1 and β2 RNAs in early postimplantation mouse embryos and uterine decidua. Mech Dev. 1992;36: 141–52.
19. Thompson NL, Flanders KC, Smith JM, Ellingworth LR, Roberts AB, Sporn MB. Expression of transforming growth factor β_3 in specific cells and tissues of adult and neonatal mice. J Cell Biol. 1989;108:661–9.
20. Akhurst RJ, Fitzpatrick DR, Fowlis DJ, Gatherer D, Millan FA, Slager HG. The role of TGF-betas in mammalian development and neoplasia. Mol Reprod Dev. 1992;32:127–35.
21. Wang X-F, Lin HY, Ng-Eaton E, Downward J, Lodish HF, Weinberg RA. Expression cloning and characterization of the TGFβ type III receptor. Cell. 1991;67:797–805.
22. Lin HY, Wang X-F, Ng-Eaton E, Weinberg RA, Lodish HF. Expression cloning of the TGFβ Type II receptor, a functional transmembrane serine/threonine kinase. Cell. 1992;68:775–85.
23. Kochhar DM. Teratogenic activity of retinoic acid. Acta Patol Microb Scand. 1967;70:398–404.
24. Schardein JL. Drugs as teratogens. Boca Raton, Florida: CRC Press; 1976:237.
25. Kochhar DM. Studies on vitamin A-induced teratogenesis effects on embryonic mesenchyme and epithelium and on incorporation of ^{3}H-thymidine. Teratology. 1968;1:299–310.
26. Saunders JW, Gasseling MT. Ectodermal–mesenchymal interactions in the origin of limb symmetry. In: Fleischmayer R, Billingham RE, eds. Epithelial-mesenchymal interactions. Baltimore, Maryland: Williams and Wilkins; 1968:78–97.
27. Tickle C, Alberts BM, Lee J, Wolpert L. Local application of retinoic acid to the limb bud mimics the action of polarizing region. Nature. 1982;296:564–5.
28. Tickle C, Lee J, Eichele G. A quantitative analysis of all-*trans*-retinoic acid on the pattern of chick wing development. Dev Biol. 1985;109:82–95.
29. Thaller C, Eichele G. Identification and spatial distribution of retinoids in the developing chick limb bud. Nature. 1987;327:625–8.
30. Satre MS, Kochhar DM. Elevations in the endogenous levels of the putative morphogen retinoic acid in embryonic mouse limb-buds associated with limb dysmorphogenesis. Dev Biol. 1989;133:529–36.
31. Maden M, Ong DE, Summerbell D, Chytil F. Spatial distribution of cellular protein binding to retinoic acid in the chick limb bud. Nature. 1988;335:733–5.
32. Dollé P, Ruberte E, Kastner P, Petkovich M, Stoner CM, Gudas LJ, Chambon P. Differential expression of genes encoding α, β and γ retinoic acid receptors and CRABP in the developing limbs of the mouse. Nature. 1989;342:702–5.
33. Eichele G. Retinoids and vertebrate limb pattern formation. TIG. 1989;5:246–51.
34. Mummery CL, van der Saag PT. Mediators of cell growth and differentiation. In: Hodges GM, Rowlatt C, eds. Developmental biology and cancer. New Jersey: Telford Press; [In press].
35. Darmon M. The nuclear receptor of retinoic acid. J Lipid Mediat. 1990;2:247–56.
36. Kruyt FAE, van den Brink CE, Defize LHK, et al. Transcriptional regulation of retinoic acid receptor β in retinoic acid sensitive and resistant P19 embryocarcinoma cells. Mech Dev. 1991;33:171–8.
37. Hu L, Gudas LJ. Cyclic AMP analogs and retinoic acid influence the expression of retinoic acid receptor α, β and γ mRNAs in F9 teratocarcinoma cells. Mol Cell Biol. 1990; 10:391.
38. Song S, Siu CH. Retinoic acid regulation of the expression of retinoic acid receptors in wild-type and mutant embryonal carcinoma cells. FEBS Lett. 1989;256:51.
39. Strickland S, Mahdavi V. The induction of differentiation in teratocarcinoma stem cells by retinoic acid. Cell. 1978;15: 393–403.
40. Mummery CL, Feijen A, Freund E, Shen S. Characteristics of embryonic stem cell differentiation: a comparison with two embryonal carcinoma cell lines. Cell Diff Dev. 1990; 30:195–206.
41. Evans RM. The steroid and thyroid hormone receptor super family. Science. 1988;240:889.
42. Zelent A, Mendelsohn C, Kastner P, et al. Differentially expressed isoforms of the mouse retinoic receptor β are generated by usage of two promotors and alternative splicing. EMBO J. 1991;10:71–81.
43. Durston AJ, Timmermans JPM, Hage WJ, et al. Retinoic acid causes an anteroposterior transformation in the developing central nervous system. Nature. 1989;340:140–4.
44. Sive HL, Draper BW, Harland RM, Weintraub H. Identification of a retinoic acid-sensitive period during primary axis formation in Xenopus laevis. Genes Devel. 1990;4:932–42.
45. Hilton DJ, Nicola NA, Metcalf D. Purification of a murine leukemia inhibitory factor from Krebs ascites cells. Ann Biochem. 1988;173:359–67.
46. Hilton DJ, Nicola NA, Gough NM, Metcalf D. Resolution and purification of three distinct factors produced by Krebs ascites cells which have differentiation-inducing activity on murine mycloid leukaemia cell lines. J Biol Chem. 1988; 263:9238–43.
47. Gearing DP, Gough NM, King JA, et al. Molecular cloning and expression of cDNA encoding a murine myeloid leukaemia inhibitory factor (LIF). EMBO J. 1987;6:3995–4002.
48. Gough NM, Gearing DP, King JA, et al. Molecular cloning and expression of the human homologue of the murine gene encoding myeloid leukaemia inhibitory factor. Proc Natl Acad Sci. 1988;85:2623–27.
49. Martin GR, Evans MS. Differentiation of clonal lines of teratocarcinoma cells: Formation of embryoid bodies in vitro. Proc Natl Acad Sci. 1975;72:1441.
50. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981;292:154–6.
51. Williams RL, Hilton DJ, Pease J, et al. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature. 1988;336:684–7.
52. Smith AG, Hooper ML. Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. Dev Biol. 1987;121:1–9.
53. Smith AG, Heath JK, Donaldson DD, et al. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature. 1988;336:688–90.
54. Moreau JF, Donaldson DD, Bennett F, Witer-Giannotti J, Clark SC, Wong GG. Leukaemia inhibitory factor is identical to the myeloid growth factor human interleukin for DA cells. Nature. 1988;336:690–2.
55. Pease S, Braghetta P, Gearing D, Grail D, Williams R. Isolation of embryonic stem (ES) cells in media supplemented with recombinant leukemia inhibitory factor (LIF). Dev Biol. 1990;141:344–52.
56. Pease S, Williams RL. Formation of germ-like chimeras from embryonic stem cells maintained with recombinant leukemia inhibitory factor. Exp Cell Res. 1990;190:209–11.
57. Rathjen PD, Toth S, Willis A, Heath JK, Smith AG. Differentiation inhibiting activity is produced in matrix associated and diffusible forms that are generated by alternate promoter usage. Cell. 1990;62:1105–14.
58. Conquet F, Brûlet P. Developmental expression of myeloid leukemia inhibitory factor gene in preimplantation blasto-

cysts and in extraembryonic tissue of mouse embryos. Mol Cell Biol. 1990;10:3801–5.
59. Bhatt H, Brunet LJ, Stewart CL. Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. 1991;88:11408–12.
60. Hilton DJ, Nicola NA, Metcalf D. Specific binding of murine leukemia inhibitory factor to normal and leukemic monocytic cells. Proc Natl Acad Sci. 1988;85:5977.
61. Mummery CL, van Achterberg TAE, van den Eijnden-van Raaij AJM, et al. Visceral endoderm-like cell lines induce differentiation of murine P19 EC cells. Differentiation. 1991;46;51–60.
62. van den Eijnden-van Raaij AJM, van Achterberg T, van der Kruijssen CMM, et al. Differentiation of aggregated P19 EC cells is induced by a novel visceral-endoderm specific FGF-like factor and inhibited by activin A. Mech Dev. 1990;33:157–65.
63. Slager HG. The role of TGFβ_2 in embryonic development [Thesis]. The Netherlands: University of Utrecht; 1992.
64. Hierck BP, Thorsteinsdottir S, Niessen CM, et al. Variants of the laminin receptor in early murine development: distribution, molecular cloning and chromosomal localization of the mouse integrin α_6 subunit. Cell Adh Comm. [In press].
65. Grabel LB, Watts TD. The role of extracellular matrix in the migration and differentiation of parietal endoderm from teratocarcinoma embryoid bodies. J Cell Biol. 1987;105: 441–8.
66. Hogan B, Costantini F, Lacy E. Manipulating the mouse embryo. Cold Spring Harbor; 1986:50.